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Practical Synthesis of Cyclic Bis(3′–5′)diadenylic Acid (*c*-di-AMP)

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Cyclic bis(3′–5′)diadenylic acid (*c*-di-AMP) (Chart 1), recently identified as a second messenger monitoring DNA integrity during sporulation in the soil bacterium *Bacillus subtilis*, was synthesized on an 80 μmol scale by a combination of the phosphoramidite and phosphotriester methods using a commercially available adenosine phosphoramidite as starting material. An artificial analog 2′-bis(*tert*-butyldimethylsilyl)-*c*-di-AMP was also obtained by our procedure.

Recently, cyclic bis(3′–5′)diadenylic acid (*c*-di-AMP) was discovered from the soil bacterium *Bacillus subtilis*[1,2] and was identified as a second messenger monitoring DNA integrity during sporulation.[3] *B. subtilis* possesses DNA integrity scanning protein (DisA), which regulates generation of *c*-di-AMP: at the onset of sporulation, DisA produces *c*-di-AMP from ATP, whereas generation of *c*-di-AMP is stopped with interruption of sporulation when DisA detects branched DNA. DisA has a diadenylate cyclase (DAC) domain able to produce *c*-di-AMP. Because the DAC domain is widely present in bacteria and archaea even in some nonsporulating species, *c*-di-AMP might act as a second messenger in various ways. For example, *c*-di-AMP induces a type I interferon.[4] Moreover, *c*-di-AMP is structurally related to cyclic bis(3′–5′)diguanylic acid (*c*-di-GMP),[5] which controls cellulose synthesis,[5] biofilm formation, motility, and virulence factor production in a variety of bacteria.[6–8] Thus, comparison of the biological activities of *c*-di-AMP with those of *c*-di-GMP is a subject of interest, and has been performed to some degree in studies of riboswitch recognition[9] and adjuvant activities.[10] To investigate the unknown biological activities of *c*-di-AMP, a sufficient amount (ideally, 10 μmol for a series of experiments) of this molecule is necessary. However, the amount of *c*-di-AMP obtained from bacteria is limited. Furthermore, *c*-di-AMP is commercially available in units of 0.1 μmol, although, it is fairly expensive.[11] Therefore, it is very important to establish a practical synthesis of *c*-di-AMP.

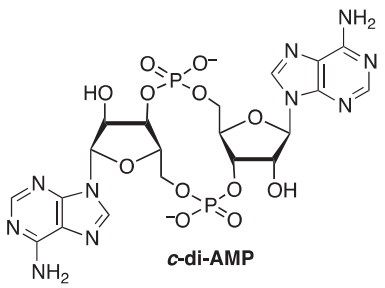
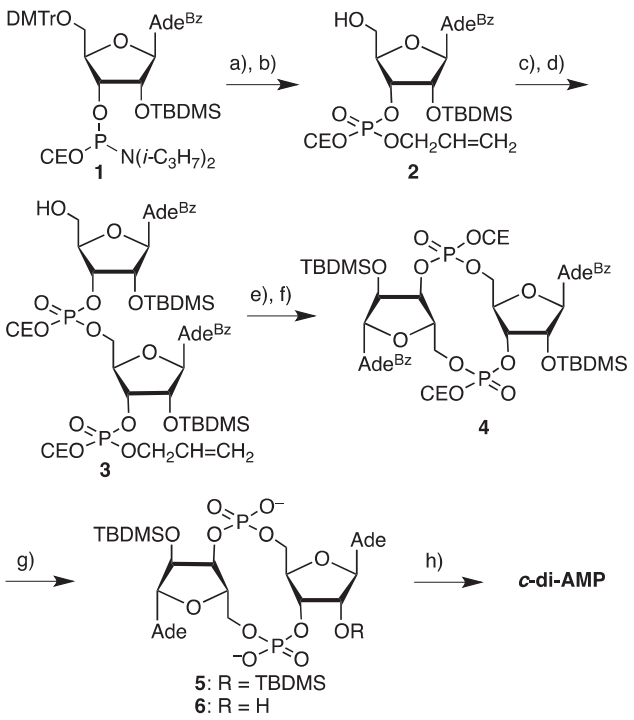


Chart 1.

Although *c*-di-AMP was suggested to be formed as a side product in some reactions[12–14] such as homopolymerization of adenosine 3′-monophosphate,[13,14] reports on target-oriented synthesis have been limited. So far, *c*-di-AMP has been synthesized by the phosphotriester method,[15,16] and by the construction of a cyclic sugar backbone and subsequent introduction of the protected adenine.[17] However, these methods consist of multistep reactions, which cannot provide a sufficient amount of *c*-di-AMP. We therefore synthesized *c*-di-AMP by a method employing phosphoramidite and phosphotriester approaches, which had already been applied to a large-scale synthesis of *c*-di-GMP by Hyodo and Hayakawa.[18] In addition, we identified and characterized the hydrophobic analog 2′-*O*-bis(*tert*-butyldimethylsilyl) (TBDMS)-*c*-di-AMP (**5**) (Scheme 1), which it is hoped will promote the biological activities of *c*-di-AMP, based on the finding that 2′-*O*-bis-TBDMS-*c*-di-GMP showed a stronger inhibitory effect than *c*-di-GMP in



Scheme 1. *Reagents and conditions*: a) 1) allyl alcohol, imidazolium perchlorate (IMP), molecular sieves 3 Å (MS 3 Å), CH_3CN, rt, 2) *t*-C_4H_9OOH/toluene, rt; b) $CHCl_2COOH$, CH_2Cl_2, 0 °C; c) 1) **1**, IMP, MS 3 Å, CH_3CN, rt, 2) *t*-C_4H_9OOH/toluene, rt; d) $CHCl_2COOH$, CH_2Cl_2, 0 °C; e) NaI, acetone, reflux; f) 2,4,6-triisopropylbenzenesulfonyl chloride, *N*-methylimidazole, 28 °C; g) concd aq. NH_3–CH_3OH (1:1 v/v), 50 °C; h) $(C_2H_5)_3N \cdot 3HF$, rt.

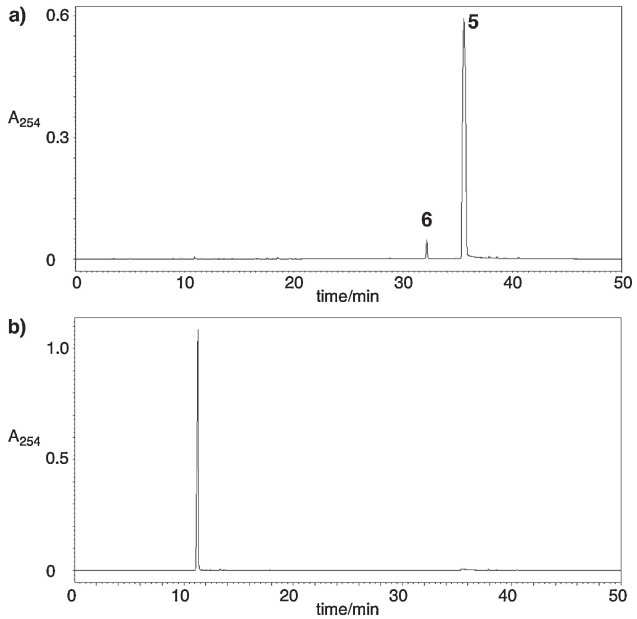


Figure 1. Reverse-phase HPLC profiles of (**a**) a mixture of **5** and **6**, and (**b**) purified *c*-di-AMP. For details of HPLC conditions, see Figure S1.[26]

Anaplasma phagocytophilum infection of human myelocytic leukemia HL-60 cells.[19]

We synthesized *c*-di-AMP according to Scheme 1 by use of the commercially available adenosine cyanoethyl (CE) phosphoramidite **1** as starting material. The amidite **1** was condensed with allyl alcohol by the aid of imidazolium perchlorate (IMP)[20] in the presence of molecular sieves 3 Å (MS 3 Å)[21] in acetonitrile. The resulting phosphite triester was oxidized with *tert*-butyl hydroperoxide[22,23] to give the corresponding phosphotriester, which was converted into the nucleotide **2** by deprotection of the 5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) group under acidic conditions. The overall yield of **2** from **1** was 96%. This three-step procedure consisting of condensation, oxidation, and detritylation was safely applied to a combination of the amidite **1** and the nucleotide **2** to give the protected linear dimer **3** in 82% yield as shown in steps c and d. Thereafter, the allyl group on the 3′-terminal phosphate of **3** was deprotected by sodium iodide.[18] The linear dinucleotide 3′-phosphodiester thus obtained was cyclized by a mixture of 2,4,6-triisopropylbenzenesulfonyl chloride and *N*-methylimidazole in THF under high-dilution conditions (substrate concentration: ca. 6 mM) to give the fully protected *c*-di-AMP **4** in 57% overall yield from **3**.[24] This yield was in the range of reported values (40–87%) in the synthesis of related compounds.[18,25]

Subsequently, benzoyl (Bz) and cyanoethyl (CE) protecting groups of **4** were removed by a mixture of concentrated aqueous ammonia and methanol to afford the bis TBDMS-protected *c*-di-AMP **5** and the mono TBDMS-protected *c*-di-AMP **6** in a ratio of 96:4 in 97% yield. Figure 1a shows the reverse-phase HPLC profile of the crude sample, indicating that partial deprotection of the TBDMS group occurs in step g (Scheme 1).

Finally, removal of the TBDMS groups of **5** and **6** was conducted in an 89 µmol scale by treatment of triethylamine trihydrofluoride to give *c*-di-AMP in 91% yield. Figure 1b shows the HPLC profile of *c*-di-AMP obtained after purification by reverse-phase preparative HPLC. The retention time of synthetic *c*-di-AMP was found to be 11.3 min, which was identical to that of the authentic sample (Figure S1 in Supporting Information (SI)[26]). Moreover, the synthetic and authentic samples showed the same fragmentation patterns in ESI tandem mass measurements (Figure S2 in SI[26]).

In summary, we synthesized *c*-di-AMP and its hydrophobic analog 2′-*O*-bis-TBDMS-*c*-di-AMP from the adenosine phosphoramidite in 40% and 44% overall yields, respectively. Our method can provide sufficient amounts of *c*-di-AMP for implementation of a variety of biological activities, and the results will be reported in due course.

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26 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

